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NR2C by NR2B subunit exchange in juvenile mice affects emotionality and 5-HT in the frontal cortex

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The *N*-methyl-D-aspartate receptor (NMDA-R) has been *inter alia* implicated in synaptic plasticity, brain development and emotional processes. The NMDA-R is a multiprotein complex composed of NR1, NR2 and/or NR3 subunits. We generated NR2C-2B mutant mice in which an insertion of NR2B cDNA into the gene locus of the NR2C gene replaced NR2C by NR2B expression throughout the brain. This NR2C-2B mutant was used to examine whether an NMDA-R subunit exchange in juvenile mice would affect emotional behaviors and acetylcholine (ACh), dopamine (DA) and serotonin (5-HT) content in the frontal cortex (FC) and brain structures, which are part of the brain defense system, such as the periaqueductal grey matter (PAG). Juvenile, 1-month-old NR2C-2B mice showed increased open arm avoidance in the elevated plus-maze and increased fear-induced immobility. In terms of brain neurochemistry, NR2C-2B mice showed an increase in 5-HT levels in the FC at the age of 2 months. A correlational analysis revealed that mice with low open arms avoidance had high levels of ACh in the PAG but reduced 5-HT levels in the FC. Animals which showed high levels of fear-induced immobility also had high levels of 5-HT in the FC. These results suggest that the replacement of subunit NR2C by NR2B in juvenile mice increases anxiety- and fear-related behaviors possibly due to changes in FC-5-HT and PAG-ACh levels.

Keywords: Acetylcholine, elevated plus-maze, gene substitution graded anxiety test, NR2B, NR2C, serotonin

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N-methyl-D-aspartate receptors (NMDA-R) have been implicated in brain maturation, neuroplasticity, emotionality and memory processes (Monyer *et al.* 1994; Mori & Mishina 1995; Morris *et al.* 1990; Wiley *et al.* 1995). The functional characteristics of multiprotein NMDA-R complexes are determined by their subunit composition. Native NMDA-R are composed of an obligatory NR1 and at least of one of four NR2 subunits (Monyer *et al.* 1994). These subunits are encoded by separate genes, which show distinct developmental, brain-regional, cellular and subcellular expression patterns (Das *et al.* 1998; Hollmann & Heinemann 1994). The NMDA-R subunit NR2C is expressed after birth and reaches adult levels within the first month (Goebel & Poosch 1999; Zhong *et al.* 1995). The NR2C mRNA and protein is predominantly expressed in the cerebellum (Zhong *et al.* 1995) but is, in lower levels, also present in the hippocampus (Rafiki *et al.* 2000), frontal cortex (Kuehl-Kovarik *et al.* 2000), amygdala (Karst *et al.* 2002), hypothalamus (Goebel & Poosch 1999) and superior and inferior colliculi (COL) (Goebel & Poosch 1999). Mice in which the coding sequence of the NR2C gene was replaced by one of the NR2B gene (NR2C-2B mice), while the regulatory sequences of the NR2C gene were kept functional (Schlett *et al.* 2004), express the NR2B subunit instead of the NR2C subunit throughout the brain. Although, changes in cerebellar microarchitecture and electrophysiology were already evident from the second postnatal week on, motor deficits appeared not until 4 months of age in NR2C-2B mice (Schlett *et al.* 2004). Similarly, although acetylcholine levels of 4-month-old NR2C-2B mice were increased in the frontal cortex and in the amygdala, at the age of 2 months these mice showed no differences compared to controls in the open-field, in a test of object recognition and in the elevated plus-maze (EPM) (De Souza Silva *et al.* 2006).

The role of the NMDA-R in unconditioned anxiety has been mainly investigated by means of subunit non-selective antagonists. Injections of the non-selective antagonist 2-amino-7-phosphonoheptanoic acid (AP7) into the dorsomedial hypothalamus of rats reduced exploratory behavior in the EPM but failed to produce an anxiolytic effect (Jardim & Guimaraes 2004). However, AP7 increased exploration of open arms in the EPM when microinjected into either the dorsolateral or ventrolateral periaqueductal grey (Molchanov & Guimaraes 2002). Dizocilpine given intraperitoneally to rats increased exploration of an EPM and reduced open arm avoidance (Fraser *et al.* 1997). Systemic administration of the competitive NMDA-R antagonist, 2*R*,4*R*,5*S*-2-amino-4,5-(1,2-cyclohexyl)-7-phosphono-heptanoic acid induced an anxiolytic behavioral profile in the EPM (Wiley *et al.* 1995). In line with the latter results, NMDA injections decreased open arm exploration in mice (Vasar *et al.* 1993). In mice, ifenprodil, a selective antagonist at NMDA-R featuring the NR2B subunit, has been reported to induce anxiolysis in an EPM (Fraser *et al.* 1996) but had no effect in the graded anxiety test (GAT)

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(Dere *et al.* 2003). Dextromethorphan, a weakly NR2C-selective NMDA-R antagonist (Monaghan & Larsen 1997), dose-dependently induced both anxiolytic and anxiogenic effects together with motor and sensory side-effects in the GAT (Dere *et al.* 2003).

In the present study, we assessed the effects of the NR2C-2B subunit exchange in 1-month-old juvenile mice on emotional behaviors and acetylcholine (ACh), dopamine (DA) and serotonin (5-HT) content in brain regions involved in anxiety-related and defensive behaviors, such as the frontal cortex (FC), amygdala (A), hypothalamus (HYP), periaqueductal grey matter (PAG) and COL (Brandao *et al.* 2003). Prior to the assessment of EPM, GAT and fear-induced escape behavior, we subjected the NR2C-2B mice to a motor test battery in order to exclude possible motor deficits.

Methods

Animals

Generation of the NR2C-2B subunit exchange mice and verification of successful subunit exchange by *in situ* hybridization and western blotting methods, as well as electrophysiological and anatomical examinations of the NR2C-2B mice was described previously (Schlett *et al.* 2004). Recombinant embryonic stem cells, (RW4) derived from 129X1/SvJ mouse blastocysts (Incyte Genomics, Wilmington, DE, USA), were injected into C57BL/6J blastocysts and subsequently transferred to B6CBF1 animals (RCC Ltd., Wüllinsdorf, Switzerland). Animals were backcrossed for at least six generations into C57BL/6J background. Homozygous receptor exchange mice and their non-mutant littermates were obtained from breeding heterozygous NR2C-2B mice. One-month-old male NR2C-2B subunit exchange ($n = 10$) and control mice ($n = 15$) were used. They were single-housed in standard Makrolon cages and had continuous access to food and tap water. The animals were maintained on a 12 h light/dark cycle and were tested during the light phase between $9^{\underline{00}}$ and $16^{\underline{00}}$. The order of behavioral testing was (1) motor test battery, (2) elevated plus-maze, (3) graded anxiety test and (4) fear-induced escape behavior. At the age of 2 months the animals were sacrificed and their brains neurochemically examined. All experiments were performed according to the guidelines of the German Animal Protection Law and were approved by the North Rhine Westphalia state authority.

In situ hybridization

In situ hybridization was performed as previously described (Klein *et al.* 1998). In short, whole brains were taken from NR2C-2B mutant and wild type mice and frozen on dry ice. Twenty-micrometer sections were cut using a Leica cryostat at −25°C, placed on silanized glass slides, fixed with 4% paraformaldehyde in phosphate-buffered saline and stored in 70% ethanol at 4°C. Antisense 35S-labeled probes were prepared from linearized templates using the T3/T7 *in vitro* transcription system (Ambion, Austin, TX, USA). Sense transcripts were used as controls. Hybridized slides were dipped with LM-1 photo emulsion (Amersham-Pharmacia, Germany) following exposition for 2 weeks at 4°C. Slides were developed according to the manufacturer's recommendations and analyzed in dark field microscopy.

Western blot analysis

Protein extracts from the hippocampus and cortex were prepared by homogenization in 8–10 volumes of ice-cold buffer containing 0.32 M sucrose, 1 mM NaHCO3, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$ and protease inhibitors (0.5 mM phenylmethylsulfonylfluoride, 10 μg/ml leupeptine, 2 μg/ml aprotinin and 10 μM bestatin), using a Potter homogenizer with 12 strokes at 800 rpm. Brain homogenates were centrifuged at 1400***g*** for 10 min at 4°C, and supernatants were used for Western blot analysis. Protein content of the samples was determined using the BioRad Bradford reagent (BioRad, Germany). Fifty micrograms protein per lane were loaded, separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane (Hybond ECL extra, Amersham, Germany). Primary antibodies were used overnight at 4°C as follows: anti-NR1 (rabbit, 1:500, Chemicon, Temecula, CA, USA), anti-NR2A (rabbit, 1:3000, Upstate Biotech, Lake Placid, NY, USA), anti-NR2B (BM 1B3.3B6, mouse, 1:10 000; Laurie *et al.* 1997) and anti-βIII-tubulin (TUJ1, mouse, 1:5000, BAbCo, Richmond, CA, USA). Blots were further incubated with anti-rabbit or anti-mouse IgGs conjugated with horse radish peroxidase (Pierce, Rockford, IL, USA) at a dilution of 1:10 000 for 1 h. Antibody binding was visualized using the enhanced chemiluminescence detection system (Amersham).

Motor test battery

Pole-test

This test measures motor co-ordination behavior. A 50 cm high, gauze-muffled vertical pole with a cork ball on top was used. Animals were placed with their head upward below the cork ball, and the latency to (a) turn downward and (b) climb to the floor (seconds) was scored with a cut-off maximum of 2 min.

Wire brachiation test

The animals were further evaluated regarding limb muscle and/or grip strength. A 2 mm thin wire of 85 cm length was horizontally clamped between two platforms of 22 cm height each. The mice were approximated to the middle of the wire and released after they grasped the wire with the forepaws. The time the animals needed to reach one of the platforms was recorded with cut-off after 60 seconds had elapsed.

Open-field

In order to examine spontaneous motor activity in an open-field, the mice were exposed to an open-field on two consecutive days. The open-field apparatus was a rectangular chamber (29 × 29 × 40 cm) made of grey PVC. The animals received one 5 min trial a day. The digitized image of the path taken by each animal was stored and analyzed post hoc with a semi-automated analysis system (Etho-Vision®, Noldus, The Netherlands). The number of times an animal stood on its hind legs with forelegs in the air or against the wall and the distance in meters an animal moved was scored by an experienced observer.

Y-maze continuous alternation

This task utilizes the congenital tendency of rodents to frequent places not visited quite recently, when allowed to choose freely among respective alternatives. The Y-maze was made of transparent Plexiglas with three arms each 8 cm wide, 35 cm long, with walls of 10 cm height and an open roof, radiating from a triangle-shaped central platform. The following parameters were recorded during a single 8 min session: (1) entries, the number of times an animal entered an arm with all four paws; (2) triplets, the number of consecutive choices of all three arms, without re-entries during the last three choices irrespective of the order of the chosen arms; (3) alternation-index computed as number of triplets divided by the total number of entries minus 2.

Emotionality

Elevated plus-maze

The apparatus consisted of two open arms (29 × 5 cm) and two non-transparent walled arms (29 × 5 × 15 cm) with an open roof and a white floor, arranged around a central platform (5 × 5 cm) so that the two arms of each type were opposite to each other. It was elevated to a height of 40 cm. The mice were placed on the central platform facing one of the walled arms and were observed for 5 min, during which the

time spent in the open and walled arms as well as in the central platform were measured.

Graded anxiety test
A modified elevated plus-maze was used. The modifications were as follows: one walled arm was transparent and had a white floor, the other was opaque-grey with a black floor. One open arm had a white floor, while the other had a black one. The mice were placed on the central platform facing one of the walled arms and were observed for 5 min, during which the time spent in the four compartments and the central platform was measured.

Fear-induced immobility
Animals were placed on the end of an open arm of the modified elevated plus-maze, with the head of the animal pointing away from the central platform. Each animal received four trials (two starts from the black arm and two from the white, quasi-randomized across groups) with an inter-trial interval of 60 seconds. The latency to escape (seconds) to one of the walled arms was measured.

Neurochemistry
5-HT, DA and ACh levels were assessed in different areas of the brain. The animals were sacrificed by cervical dislocation followed by decapitation; their brains were quickly removed and placed in an ice-cold brain matrix. Coronal sections were made following landmarks on the base of the brain, and the FC, A, HYP, PAG and COL were dissected out bilaterally onto an ice-cold platform. Thereafter, the brain tissue was weighed, homogenized in ice-cold 0.5 N perchloric acid containing ethylhomocholine as an internal standard, centrifuged, filtered and kept at -70°C until being analyzed using a procedure similar to the one described by Sethy and Francis (1988). The right and left brain tissue samples were randomly analyzed either for ACh concentrations according to the procedure utilized by Damsma *et al.* (1987), except for the internal standard (Potter *et al.* 1983), or for 5-HT and DA levels using high-performance liquid chromatography with electrochemical detection (for technical details, see De Souza Silva *et al.* 1997, 2000).

Statistics
Behavioral and neurochemical data were analyzed by means of *t*-tests for independent and dependent samples. Correlational analysis of behavioral and neurochemical parameters was performed by means of Pearson correlations. *P* values given are two-tailed and were considered to be significant when $P < 0.05$.

Results

In situ hybridization
First we investigated, whether the mutant NR2C gene locus of homozygous NR2C-2B mice actually expresses NR2B by means of *in situ* hybridization. Compared to the wild type controls, the NR2B subunit mRNA expression in NR2C-2B mutant mice was increased in several brain regions at P30 (Fig. 1a,b). The most prominent increases in NR2B mRNA were evident in the cerebellum, entorhinal cortex and the neocortex of the NR2C-2B mice. NR2B mRNA was also increased in the striatum, thalamus, nucleus accumbens and hypothalamus of the mutant mice (Fig. 1a,b). These results suggest that the mutated NR2C gene locus in the NR2C-2B exchange mice indeed expressed NR2B mRNA in addition to the NR2B mRNA produced by the native NR2B gene. The strong increase in the NR2B mRNA levels in the forebrain of the mutants suggests that in the past the NR2C

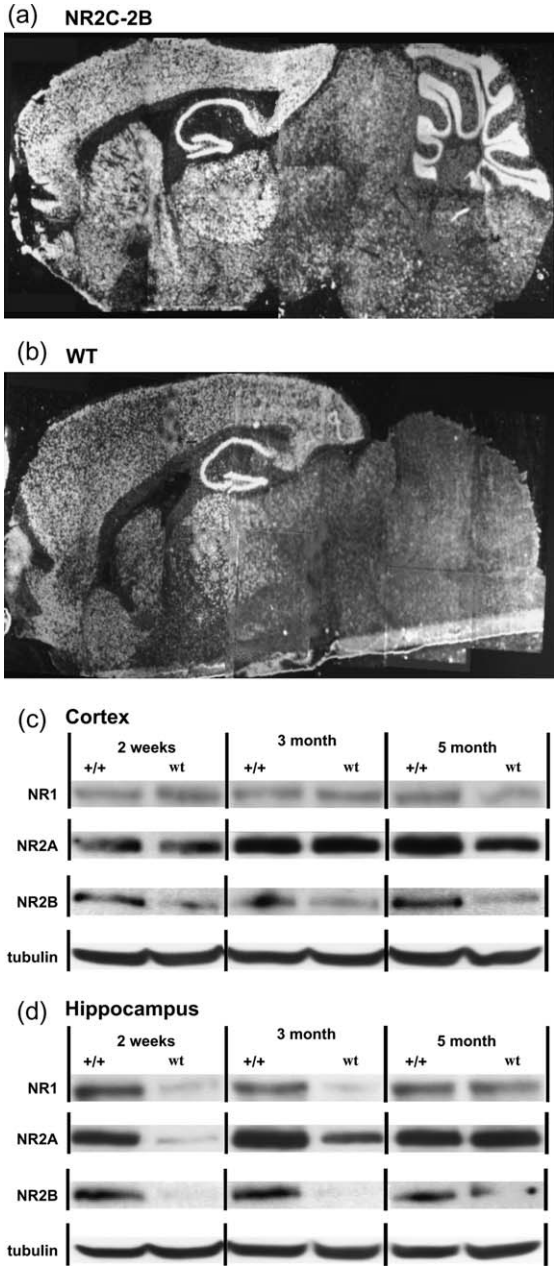

Figure 1: NR2C-2B mutants show increased staining for NR2B mRNA in the forebrain and cerebellum. (a) *In situ* hybridization for the NR2B mRNA in brain sections prepared from 30-day-old homozygous NR2C-2B mice. (b) *In situ* hybridization for the NR2B mRNA in brain sections prepared from 30-day-old wild type mice. *NMDA-R subunits protein expression in the cortex and hippocampus of NR2C-2B and wild type mice at ages of 2 weeks, 3 and 5 months.* (c) Detection of NR1, NR2A and NR2B subunit protein in cortex lysates of NR2C-2B mutants (+/+) and wild type mice (wt). βIII-tubulin immunoblot shows the level of protein loaded in the lanes. (d) Detection of NR1, NR2A and NR2B subunit protein in hippocampus lysates of NR2C-2B mutants (+/+) and wild type mice (wt). βIII-tubulin immunoblot shows the level of protein loaded in the lanes.

subunit expression in the forebrain of wild type mice was by far underestimated.

Western blot analysis

In order to know whether the increase in NR2B mRNA also translates into increased levels of NR2B protein, we analyzed NR2B protein levels in the cortex and hippocampus at the age of 2 weeks, 3 months and 5 months in mutant and wild type mice (Fig. 1c,d). In both the cortex and hippocampus, the level of NR2B protein was strongly increased in the mutants at all time points evaluated. We also assessed the levels of NR1 and NR2A subunits in these brain regions. While the mutant mice showed similar levels of the NR1 and NR2A subunit in the cortex relative to the wild type controls, there was an increase of both NR1 and NR2A protein in the hippocampus of the NR2C-2B mice (Fig. 1c,d).

Motor test battery

The performance of NR2C-2B mice in the motor test battery, which included assessment of motor co-ordination, muscle strength, locomotory and rearing behavior, as well as exploratory behavior in a novel environment was similar to those of the controls (all $P > 0.05$; *t*-test for independent samples; Table 1).

Emotionality

Open-field

Thigmotaxis, that is the time spent in contact with the sidewalls of the open-field is sometimes used as a measure for emotionality in the open-field. We therefore assessed thigmotaxis in the NR2C-2B and control mice during the 2 days of open-field testing. However, there was no significant difference between groups evident, neither on day 1 nor on day 2 ($P > 0.05$; *t*-test for dependent samples; data not shown). High rates of defecation after exposure to a novel environment has been regarded as reflecting increased emotionality (Leppanen *et al.* 2005). Compared to the controls, the NR2C-2B mice showed significantly higher rates of defecation, measured as the number of boli deposited during the exposures to the open-field (Day 1, NR2C-2B 4.6 ± 0.78 vs. WT 2.53 ± 0.52; $P < 0.031$; Day 2, NR2C-2B 4.0 ± 0.54 vs. WT 1.67 ± 0.42; $P < 0.002$; *t*-test for dependent samples).

Elevated plus-maze

Both groups spent significantly more time in the walled compared to the open arms and the central platform ($P < 0.001$; *t*-test for dependent samples; Fig. 2a). NR2C-2B mice spent significantly less time on the open arms compared to the controls ($P = 0.030$; *t*-test for independent samples). The time spent in the walled arms and on the central platform was similar between groups ($P > 0.05$). These results suggest an anxiogenic effect of the subunit substitution in 1-month-old mice.

Graded anxiety test

Both groups showed an avoidance pattern characteristic for the GAT (Dere *et al.* 2002). Within-groups comparisons revealed that both groups spent significantly more time in the walled-opaque arm than in the walled transparent arm (NR2C-2B: $P < 0.001$, controls: $P < 0.001$; *t*-test for dependent samples; Fig. 2b). Whereas the controls had a significantly higher sojourn time on the open black compared to the open white arm (controls: $P < 0.032$), no such difference was found for NR2C-2B mice (NR2C-2B: $P > 0.05$). Actually, only 2 out of 10 NR2C-2B mice ever visited the open black arm, while the most aversive open white arm was completely avoided. On average the controls spent more time on the open white arm than the NR2C-2B mice. However, this difference failed to reach the level of statistical significance ($P = 0.088$; *t*-test for independent samples). The time spent in the remaining compartments was largely similar between groups (open black arm, $P > 0.05$; walled transparent arm, $P > 0.05$; walled-opaque grey arm, $P > 0.05$; *t*-test for independent samples). These results are in line with the results of the standard EPM suggesting an anxiogenic effect of the gene intervention in juvenile mice around P28.

Fear-induced immobility

We next investigated whether an escape from aversive stimulation is equally altered in NR2C-2B mice. On the first and last trials NR2C-2B mice were significantly slower in escaping to a walled arm relative to the controls (trial 1: $P = 0.029$, trial 2: $P > 0.05$, trial 3: $P < 0.05$, trial 4: $P = 0.046$; Fig. 2c), suggesting that escape behavior is altered in NR2C-2B mice.

Neurochemistry

Although not statistically significant, the NR2C-2B mice had lower ACh levels in the PAG compared to the controls ($P = 0.083$; *t*-test for independent samples; Table 2). ACh concentrations in the other brain areas assessed were similar between groups (all $P > 0.05$). 5-HT concentrations in the FC were increased in the NR2C-2B mice relative to controls ($P < 0.001$; Table 3). In the other brain areas assessed, 5-HT levels were similar between groups (all $P > 0.05$). Compared to control mice, DA levels of NR2C-2B mice were somewhat lower in the PAG ($P = 0.058$; Table 4) but comparable to controls in the remaining brain regions considered (all $P > 0.05$).

Correlations

Neurochemical and behavioral parameters, for which genotype differences were found, were subjected to a correlational analysis. Animals which showed high sojourn times on the open arms of the EPM also showed high levels of ACh in the PAG ($r = 0.48$; $P = 0.016$; Pearson correlation) but reduced levels of 5-HT in the FC ($r = -0.47$; $P = 0.017$). In contrast, DA levels in the PAG were not correlated with the time spent on the open arms ($r = 0.09$; $P > 0.05$). Animals which showed high latencies to escape to the walled arms in the fear-induced immobility test also showed high levels of 5-HT in the FC ($r = 0.45$; $P = 0.023$). No such correlation with fear-induced immobility was found for ACh ($r = -0.31$; $P > 0.05$) and DA levels in the PAG ($r = -0.24$; $P > 0.05$). These results suggest that the altered emotional behavior of NR2C-2B mice

Table 1: Similar performance of NR2C-2B subunit exchange and wild type mice in a motor test battery

	Pole-test		Wire-test	Open-field locomotion (m)		Open-field rearings		Y-maze		
Genotype	180° turn (seconds)	Latency (seconds)	Latency (seconds)	Day 1	Day 2	Day 1	Day 2	Entries	Triplets	Alternation
NR2C-2B	14.9 ± 7.2	47.8 ± 16.6	26.0 ± 5.8	12.0 ± 1.0	11.1 ± 0.4	14.7 ± 3.4	15.5 ± 1.4	29.4 ± 1.9	14.9 ± 1.7	53.2 ± 2.8
WT	10.5 ± 3.4	46.1 ± 14.5	29.3 ± 5.0	13.3 ± 0.8	12.2 ± 0.9	20.3 ± 2.4	22.5 ± 3.6	32.5 ± 3.2	17.2 ± 1.7	57.3 ± 2.4

is related to the observed changes in 5-HT levels in the FC and ACh concentrations in the PAG.

Discussion

Mice in which the coding sequence of the NR2C gene was replaced by one of the NR2B gene, while the regulatory sequences of the NR2C gene were kept functional (Schlett *et al.* 2004), expressed the NR2B subunit instead of the NR2C subunit throughout the brain. The aim of the present study was to assess the effects of the NR2C-2B subunit exchange in juvenile mice on emotional behavior and several neurotransmitters in brain regions involved in anxiety-related and defensive behaviors. Prior to the assessment of EPM, GAT and fear-induced immobility, we subjected the NR2C-2B mice to a motor test battery. NR2C-2B mice showed unimpaired motor co-ordination, muscle strength, locomotory and rearing behavior, as well as exploratory behavior in a novel environment. The NMDA-R NR2C by NR2B subunit exchange increased unconditioned anxiety and modified fear-induced immobility in 1-month-old mice. At the age of 2 months, the NR2C-2B mice also showed a significant increase in 5-HT levels in the FC. A correlational analysis revealed that mice which showed low open arm avoidance displayed high levels of ACh in the PAG but reduced 5-HT levels in the FC. Animals which showed high levels of fear-induced immobility also showed high levels of 5-HT in the FC.

The EPM test measures unconditioned anxiety and is sensitive to anxiolytic drugs such as benzodiazepines (Pellow & File 1986) and is widely used for emotional phenotyping of mouse mutants (Belzung & Griebel 2001). Here, increases and decreases in the time spent on the open arms are interpreted as anxiolytic and anxiogenic effects, respectively. In the EPM the NR2C-2B mice spent less time on the open arms and completely avoided the most aversive open bright arm in the GAT, suggesting an anxiogenic effect of the subunit replacement in juvenile mice. Because the NR2C-2B mice showed unimpaired motor performance in a motor test battery, these effects cannot be explained by motor impairments.

Sudden exposure to an aversive situation such as a predator, electric shock or to an unfamiliar environment induces motor paralysis in human and nonhuman juveniles (Kaada 1987). Because the NR2C-2B mice showed higher open arm avoidance, we investigated whether they would show a fear-induced immobility after a sudden placement on an open arm. Relative to the controls, the escape latency of NR2C-2B mice was increased during the first trial, suggesting that NR2C-2B mice were indeed immobile, when suddenly placed on an open arm. During the subsequent trials the NR2C-2B mice obviously learned to cope with this situation, as reflected by the decreased escape latencies on trials 2–4. However, escape latencies of NR2C-2B mice were again higher on the fourth trial compared to controls. These results are in line with evidence showing that NMDA-R in the PAG are involved in defensive behaviors after aversive stimulation (Bittencourt *et al.* 2004).

We next evaluated whether these behavioral effects are associated with changes in neurotransmitter contents in brain regions proposed to be part of a *brain defense system* (Brandao *et al.* 2003) and the FC, which was also implicated in emotional behaviors (Shah & Treit 2004; Shah *et al.* 2004). Here, the NR2C-2B mice showed a significant increase in 5-HT levels in the FC and on average decreased ACh and DA concentrations in the PAG grey.

The neurochemical changes in the PAG, although not statistically significant, are nevertheless interesting because they are in line with evidence showing that NMDA-R antagonists microinjected into the PAG modulate anxiety-related behaviors in the EPM (Molchanov & Guimaraes 2002) and that cholinergic (Graeff 1994) and dopaminergic systems (Jenck *et al.* 1989) modulate defensive behaviors mediated by the PAG. Furthermore, it has been shown that changes in emotional behavior go along with changes in FC-5-HT either in terms of tissue content or metabolism in several mouse mutants, such as endothelial nitric oxide synthase (Dere *et al.*

Table 2: Mean (±SEM) concentration (pmol/mg) of acetylcholine in indicated brain areas of NR2C-2B subunit exchange and wild type mice

Genotype	Frontal cortex	Amygdala	Hypothalamus	Periaqueductal grey	Colliculi
NR2C-2B	6.2 ± 0.2	9.2 ± 0.7	7.3 ± 0.3	12.2 ± 0.7*	13.0 ± 1.0
WT	5.6 ± 0.3	9.1 ± 0.6	7.7 ± 0.5	14.4 ± 0.8	14.0 ± 0.8

*$P < 0.1$, NR2C-2B vs. WT mice, *t*-test for independent samples.

Table 3: Mean (±SEM) concentration (pg/mg) of serotonin in indicated brain areas of NR2C-2B subunit exchange and wild type mice

Genotype	Frontal cortex	Amygdala	Hypothalamus	Periaqueductal grey	Colliculi
NR2C-2B	701.0 ± 18.0***	729.9 ± 66.7	756.7 ± 79.0	388.9 ± 105.4	616.5 ± 74.3
WT	524.9 ± 19.4	687.2 ± 31.8	625.1 ± 79.4	666.6 ± 145.7	680.1 ± 49.3

***$P < 0.001$, NR2C-2B vs. WT mice, *t*-test for independent samples.

2002; Frisch *et al.* 2000), histidine decarboxylase (Dere *et al.* 2004) and NMDA-R NR2D subunit knockout mice (Miyamoto *et al.* 2002).

In order to know whether the above neurochemical effects could account for the behavioral differences observed, a correlational analysis, including all animals tested, was performed. We found that non-anxious mice had high levels of ACh in the PAG but reduced 5-HT levels in the FC. Animals which showed fear-induced immobility had higher 5-HT concentrations in the FC.

Due to the lack of NMDA-R subunit selective agonists and antagonists, the role of single NMDA-R subunits for these emotional behaviors is still unknown. Recently, we showed that dextromethorphan, an NMDA-R antagonist, which has a higher potency to block recombinant NR1-NR2C compared to both recombinant NR1-NR2A and NR1-NR2B NMDA-R (Monaghan & Larsen 1997), dose-dependently modulates anxiety-related behavior in the GAT (Dere *et al.* 2003). The few studies assessing the effect of pharmacological NR2B blockade on unconditioned anxiety have yielded inconsistent results (Dere *et al.* 2003; Fraser *et al.* 1996).

What might be the molecular mechanism by which an NR2C-2B gene substitution induces the present effects? As expected, western blot analyses of cerebellar tissue showed that the NR2C protein is completely absent in the NR2C-2B mice (Schlett *et al.* 2004), and it is not reasonable to assume that NR2C is present in other brain areas because the DNA parts coding for the NR2C mRNA have been removed.

The NR2B mRNA and protein levels are strongly increased in the cerebellum, hippocampus and cortex of NR2C-2B mice. Furthermore, we observed increased levels of NR2B mRNA in the striatum, thalamus, nucleus accumbens and hypothalamus. The strong increase in the NR2B mRNA levels in the forebrain of the mutants suggests that wild type mice express much more NR2C in the forebrain than formerly assumed (Zhong *et al.* 1995), possibly because of the rather low sensitivity of the NR2C mRNA detection protocols used. However, it remains to be determined whether this increase in NR2B mRNA and protein expression results from the mutated NR2C gene locus and/or is due to an overexpression from the native NR2B gene locus. There was also an increase of NR1 and NR2A protein levels in hippocampal tissue of NR2C-2B mutants. The latter result suggests that an overexpression of an NR2 subunit from a native gene locus goes along with a concomitant increase in the NR1 subunit, possibly to incorporate these additional subunits into functional NMDA-R. Given that the increase in NR2B stems from the native NR2B gene locus instead of the mutated NR2C gene locus and given that these additional NR2B subunits are incorporated to NMDA-R, one would have also expected a concomitant increase in NR1 protein levels in the cerebellum and cortex, as was the case in the hippocampus. However, our western blot results suggest that, at least in the cortex, this was not the case. On the other hand, it is still possible that the additional NR2B subunits have not been incorporated into NMDA-R. Because the NR1 protein levels of NR2C-2B mice in cerebellar (Schlett *et al.* 2004) and cortical tissue was similar to the controls, one can assume that the NR2C-2B subunit exchange at the genomic level has not changed the total number of NMDA-R, at least in these brain regions. Thus, it seems reasonable to assume that the mutated NR2C gene locus expresses NR2B instead of NR2C and, given the present neurochemical and behavioral effects, that these NR2B receptors are incorporated into functional NMDA-R. Compared to NR2C-containing NMDA-R, those featuring the NR2B subunits have higher single channel conductances, a stronger voltage-dependent blockade by extracellular Mg^{2+}, a higher affinity to glutamate and are coupled to intracellular second messenger systems, which are involved in activity dependent gene expression (Cull-Candy *et al.* 2001; Kutsuwada *et al.* 1992; Monyer *et al.* 1992; Sans *et al.* 2000; Strack *et al.* 2000). Furthermore, it is known that NR1-NR2C receptors are already activated by a membrane depolarization of about −35 mV, while NR1-NR2B and NR1-NR2A receptors require a depolarization of about −25 mV to be activated (Kuner & Schoepfer 1996). Given these marked differences, it is reasonable to assume that the observed neurochemical and behavioral effects in NR2C-2B mice are due to altered properties of the accordant NMDA-R. However, because the NR2C-2B mice might not only express additional NR1-NR2B or triheteromeric NR1-NR2A/NR2B receptors in the forebrain but also lack the

Table 4: Mean (±SEM) concentration (pg/mg) of dopamine in indicated brain areas of NR2C-2B subunit exchange and wild type mice

Genotype	Frontal cortex	Amygdala	Hypothalamus	Periaqueductal grey	Colliculi
NR2C-2B	42.3 ± 4.3	316.7 ± 43.7	923.3 ± 147.2	396.9 ± 25.4*	145.7 ± 9.3
WT	45.4 ± 8.2	342.1 ± 28.9	879.8 ± 86.9	891.6 ± 230.7	136.9 ± 6.3

*$P < 0.1$, NR2C-2B vs. WT mice, *t*-test for independent samples.

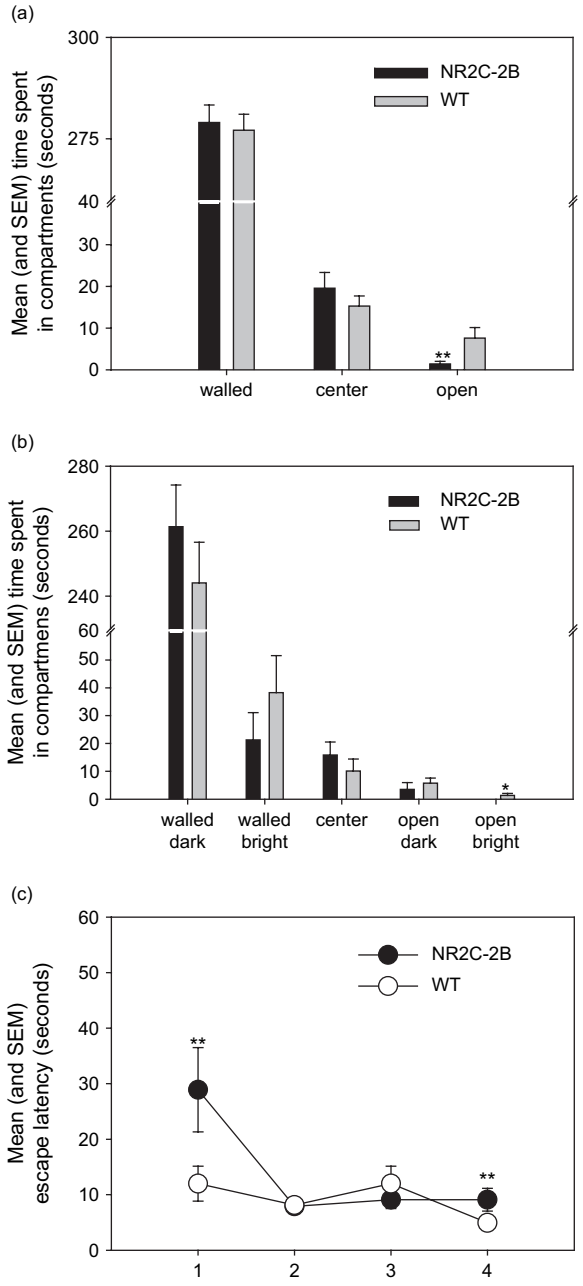

Figure 2: NMDA-R NR2C-2B subunit exchange mice show increased anxiety and fear-induced motor immobility. (a) *Elevated plus-maze.* Bars represent mean (+SEM) time spent on the central platform, open and walled arms in seconds. $^{**}P < 0.05$, *t*-test for independent samples. (b) *Graded anxiety test.* Bars represent mean (±SEM) time spent on the central platform open and walled arms in seconds. $^{*}P < 0.1$, *t*-test for independent samples. (c) *Fear-induced defensive behavior.* Circles represent mean (±SEM) escape latencies in seconds. $^{**}P < 0.05$, *t*-test for independent samples.

NR2C subunit throughout the brain, the present neurochemical and behavioral effects might also be due to the absence of NMDA-R lacking the NR2C subunit.

There was also an increase of NR1 and NR2A protein levels in hippocampal tissue of NR2C-2B mutants. Because the NR2C subunit was detected in hippocampal inhibitory interneurons, while NR2A and NR2B are mainly expressed in pyramidal cells (Ritter *et al.* 2002), the above changes might reflect an attempt to compensate for a change in inhibitory neurotransmission in the hippocampus, possibly affecting learning and memory performance of NR2C-2B mice.

Finally, it is known that the types of NR2 subunits expressed in NMDA-R determine their subcellular localization. NR2B containing NMDA-R are generally found at extrasynaptic sites (Tovar & Westbrook 1999). Thus, it is possible that in the NR2C-2B mutants, not only the functional properties of NMDA-R but also their subcellular distribution are changed, which should certainly influence the activity of the concerning cells.

In conclusion, the present results suggest that the replacement of the subunit NR2C by NR2B in juvenile mice increases anxiety and defensive behaviors, possibly due to changes in FC-5-HT and PAG-ACh levels.

References

Belzung, C. & Griebel, G. (2001) Measuring normal and pathological anxiety-like behaviour in mice: a review. *Behav Brain Res* **125**, 141–149.

Bittencourt, A.S., Carobrez, A.P., Zamprogno, L.P., Tufik, S. & Schenberg, L.C. (2004) Organization of single components of defensive behaviors within distinct columns of periaqueductal gray matter of the rat: role of N-methyl-D-aspartic acid glutamate receptors. *Neuroscience* **125**, 71–89.

Brandao, M.L., Troncoso, A.C., De Souza Silva, M.A. & Huston, J.P. (2003) The relevance of neuronal substrates of defense in the midbrain tectum to anxiety and stress: empirical and conceptual considerations. *Eur J Pharmacol* **463**, 225–233.

Cull-Candy, S., Brickley, S. & Farrant, M. (2001) NMDA receptor subunits: diversity, development and disease. *Curr Opin Neurobiol* **11**, 327–335.

Damsma, G., Westerink, B.H., De Vries, J.B., Van den Berg, C.J. & Horn, A.S. (1987) Measurement of acetylcholine release in freely moving rats by means of automated intracerebral dialysis. *J Neurochem* **48**, 1523–1528.

Dere, E., De Souza Silva, M.A., Topic, B., Fiorillo, C., Li, J.S., Sadile, A.G., Frisch C. & Huston, J.P. (2002) Aged endothelial nitric oxide synthease knockout mice exhibit higher mortality concomitant with impaired open-field habituation and alterations in forebrain neurotransmitter levels. *Genes Brain Behav* **1**, 204–213.

Dere, E., Topic, B., De Souza Silva, M.A., Srejic, M., Frisch, C., Buddenberg, T. & Huston, J.P. (2002) The graded anxiety test: a novel test of murine unconditioned anxiety based on the principles of the elevated plus-maze and light-dark test. *J Neurosci Methods* **122**, 65–73.

Dere, E., Topic, B., De Souza Silva, M.A., Fink, H., Buddenberg, T. & Huston, J.P. (2003) Antagonism of NMDAR-2A and 2B via Dextromethorphan and Ifenprodil modulates graded anxiety test performance of C57BL/6 mice. *Behav Pharmacol* **14**, 245–249.

Dere, E., De Souza Silva, M.A., Spieler, R.E., Lin, J.S., Ohtsu, H., Haas, H.L. & Huston, J.P. (2004) Changes in motoric, exploratory and emotional behaviours and neuronal acetylcholine content and 5-HT turnover in histidine decarboxylase-KO mice. *Eur J Neurosci* **20**, 1051–1058.

De Souza Silva, M.A., Mattern, C., Häcker, R., Nogueira, P.J.C., Huston, J.P. & Schwarting, R.K.W. (1997) Intranasal administration of the dopaminergic agonists L-Dopa, amphetamine and cocaine

increases activity in the neostriatum: a microdialysis study in the rat. *J Neurochem* **68**, 233–239.
De Souza Silva, M.A., Hasenöhrl, R.U., Tomaz, C., Schwarting, R.K.W. & Huston, J.P. (2000) Modulation of frontal cortex acetylcholine and behavior by injection of substance P into the ventral pallidum in the freely-moving vs. anesthetized preparation. *Synapse* **38**, 243–253.
De Souza Silva, M.A., Dolga, A., Pieri, I., Marchetti, L., Eisel, U.L.M., Huston, J.P. & Dere, E. (2006) Cholinergic cells in the nucleus basalis of mice express the *N*-methyl-D-asparate-receptor subunit NR2C and its replacement by the NR2B subunit enhances frontal and amygdaloid acetylcholine levels. *Genes, Brain and Behav* **5**, 552–560.
Fraser, C.M., Cooke, M.J., Fisher, A., Thompson, I.D. & Stone, T.W. (1996) Interactions between ifenprodil and dizocilpine on mouse behaviour in models of anxiety and working memory. *Eur Neuropsychopharmacol* **6**, 311–316.
Fraser, C.M., Fisher, A., Cooke, M.J., Thompson, I.D. & Stone, T.W. (1997) The involvement of adenosine receptors in the effect of dizocilpine on mice in the elevated plus-maze. *Eur Neuropsychopharmacol* **7**, 267–273.
Frisch, C., Dere, E., De Souza Silva, M.A., Gödecke, A., Schrader, J. & Huston, J.P. (2000) Superior water maze learning and increase in fear-related behavior in the eNOS−/− mouse together with monoamine changes in cerebellum and ventral striatum. *J Neurosci* **20**, 6684–6700.
Goebel, D.J. & Poosch, M.S. (1999) NMDA receptor subunit gene expression in the rat brain: a quantitative analysis of endogenous mRNA levels of NR1 com, NR2A, NR2B, NR2C, NR2D and NR3A. *Mol Brain Res* **69**, 164–170.
Graeff, F.G. (1994) Neuroanatomy and neurotransmitter regulation of defensive behaviors and related emotions in mammals. *Braz J Med Biol Res* **27**, 811–829.
Hollmann, M. & Heinemann, S. (1994) Cloned glutamate receptors. *Annu Rev Neurosci* **17**, 31–108.
Jardim, M.C. & Guimaraes, F.S. (2004) Role of glutamate ionotropic receptors in the dorsomedial hypothalamic nucleus on anxiety and locomotor behavior. *Pharmacol Biochem Behav* **79**, 541–546.
Jenck, F., Broekkamp, C.L. & Van Delft, A.M. (1989) Effects of serotonin receptor antagonists on PAG stimulation induced aversion: different contributions of 5HT1, 5HT2 and 5HT3 receptors. *Psychopharmacology (Berl.)* **97**, 489–495.
Kaada, B. (1987) The sudden infant death syndrome induced by ''the fear paralysis reflex'? *Med Hypotheses* **22**, 347–356.
Karst, H., Nair, S., Velzing, E., Rumpff van Essen, L., Slagter, E., Shinnick-Gallagher, P. & Joels, M. (2002) Glucocorticoids alter calcium conductances and calcium channel subunit expression in basolateral amygdala neurons. *Eur J Neurosci* **16**, 1083–1089.
Klein, M., Pieri, I., Uhlmann, F., Pfizenmaier, K. & Eisel U. (1998) Cloning and characterization of promoter and 5′-UTR of the NMDA receptor subunit epsilon 2: evidence for alternative splicing of 5′-non-coding exon. *Gene* **208**, 259–269.
Kuehl-Kovarik, M.C., Magnusson, K.R., Premkumar, L.S. & Partin, K.M. (2000) Electrophysiological analysis of NMDA receptor subunit changes in the aging mouse cortex. *Mech Ageing Dev* **115**, 39–59.
Kuner, T. & Schoepfer, R. (1996) Multiple structural elements determine subunit specificity of Mg2+ block in NMDA receptor channels. *J Neurosci* **16**, 3549–3558.
Kutsuwada, T., Kashiwabuchi, N., Mori, H., Sakimura, K., Kushiya, E., Araki, K., Meguro, H., Masaki, H., Kumanishi, T., Arakawa, M. & Mishina, M. (1992) Molecular diversity of the NMDA receptor channel. *Nature* **358**, 36–41.
Laurie, D.J., Bartke, I., Schoepfer, R., Naujoks, K. & Seeburg, P.H. (1997) Regional, developmental and interspecies expression of the four NMDAR2 subunits, examined using monoclonal antibodies. *Mol Brain Res* **51**, 23–32.
Leppanen, P.K., Ewalds-Kvist, S.B., Selander, R.K. (2005) Mice selectively bred for open-field thigmotaxis: life span and stability of the selection trait. *J Gen Psychol* **132**, 187–204.
Miyamoto, Y., Yamada, K., Noda, Y., Mori, H., Mishina, M. & Nabeshima, T. (2002) Lower sensitivity to stress and altered monoaminergic neuronal function in mice lacking the NMDA receptor epsilon 4 subunit. *J Neurosci* **22**, 2335–2342.
Molchanov, M.L. & Guimaraes, F.S. (2002) Anxiolytic-like effects of AP7 injected into the dorsolateral or ventrolateral columns of the periaqueductal gray of rats. *Psychopharmacology (Berl.)* **160**, 30–38.
Monaghan, D.T. & Larsen, H. (1997) NR1 and NR2 subunit contributions to N-methyl-D-aspartate receptor channel blocker pharmacology. *J Pharmacol Exp Ther* **280**, 614–620.
Monyer, H., Sprengel, R., Schoepfer, R., Herb, A., Higuchi, M., Lomeli, H., Burnashev, N., Sakmann, B. & Seeburg, P.H. (1992) Heteromeric NMDA receptors: molecular and functional distinction of subtypes. *Science* **256**, 1217–1221.
Monyer, H., Burnashev, N., Laurie, D.J., Sakmann, B. & Seeburg, P.H. (1994) Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* **12**, 529–540.
Mori, H. & Mishina, M. (1995) Structure and function of the NMDA receptor channel. *Neuropharmacology* **34**, 1219–1237.
Morris, R.G., Davis, S. & Butcher, S.P. (1990) Hippocampal synaptic plasticity and NMDA receptors: a role in information storage? *Philos Trans R Soc Lond B Biol Sci* **329**, 187–204.
Pellow, S. & File, S.E. (1986) Anxiolytic and anxiogenic drug effects on exploratory activity in an elevated plus-maze: a novel test of anxiety in the rat. *Pharmacol Biochem Behav* **24**, 525–529.
Potter, P.E., Meek, J.L. & Neff, N.H. (1983) Acetylcholine and choline in neuronal tissue measured by HPLC with electrochemical detection. *J Neurochem* **41**, 188–194.
Rafiki, A., Bernard, A., Medina, I., Gozlan, H. & Khrestchatisky, M. (2000) Characterization in cultured cerebellar granule cells and in the developing rat brain of mRNA variants for the NMDA receptor 2C subunit. *J Neurochem* **74**, 1798–1808.
Ritter, L.M., Vazquez, D.M. & Meador-Woodruff, J.H. (2002) Ontogeny of ionotropic glutamate receptor subunit expression in the rat hippocampus. *Dev Brain Res* **139**, 227–236.
Sans, N., Petralia, R.S., Wang, Y.X., Blahos, J., Hell, J.W. & Wenthold, R.J. (2000) A developmental change in NMDA receptor–associated proteins at hippocampal synapses. *J Neurosci* **20**, 1260–1271.
Schlett, K., Pieri, I., Metzger, F., Marchetti, L., Steigerwald, F., Dere, E., Kirilly, D., Tárnok, K., Barabás, B., Varga, A.K., Gerspach, J., Huston, J.P., Pfizenmaier, K., Köhr, G. & Eisel, U.L.M. (2004) Long-term NR2B expression in the cerebellum alters granule cell development and leads to NR2A downregulation and motor deficits. *Mol Cell Neurosci* **27**, 215–226.
Sethy, V.M. & Francis, J.W. (1988) Regulation of brain acetylcholine concentration by muscarinic receptors. *J Pharmacol Exp Ther* **246**, 243–248.
Shah, A.A. & Treit, D. (2004) Infusions of midazolam into the medial prefrontal cortex produce anxiolytic effects in the elevated plus-maze and shock-probe burying tests. *Brain Res* **996**, 31–40.
Shah, A.A., Sjovold, T. & Treit, D. (2004) Inactivation of the medial prefrontal cortex with the GABAA receptor agonist muscimol increases open-arm activity in the elevated plus-maze and attenuates shock-probe burying in rats. *Brain Res* **1028**, 112–115.
Strack, S., McNeill, R.B. & Colbran, R.J. (2000) Mechanism and regulation of calcium/calmodulin-dependent protein kinase II targeting to the NR2B subunit of the N-methyl-D-aspartate receptor. *J Biol Chem* **275**, 23798–23806.
Vasar, E., Harro, J., Lang, A., Soosaar, A., Oopik, T., Koks, S., Sihver, S. & Volke, V. (1993) Anti-exploratory effect of N-methyl-D-aspartate in elevated plus-maze. *Eur Neuropsychopharmacol* **3**, 63–73.
Wiley, J.L., Cristello, A.F. & Balster, R.L. (1995) Effects of site-selective NMDA receptor antagonists in an elevated plus-maze model of anxiety in mice. *Eur J Pharmacol* **294**, 101–107.
Zhong, J., Carrozza, D.P., Williams, K., Pritchett, D.B. & Molinoff, P.B. (1995) Expression of mRNAs encoding subunits of the NMDA receptor in developing rat brain. *J Neurochem* **64**, 531–539.

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